SOME CULTURAL CONDITIONS THAT CONTROL PRODUCTION OF RORIDIN E AND SATRATOXIN H BY *STACHYBOTRYS CHARTARUM*

par I.A. EL-KADY & M.H. MOUBASHER*

SUMMARY. — *Stachybotrys chartarum* (isolate No 128) successfully produced satratoxin H and one of the highly cytotoxic compounds, roridin E. Biosynthesis of both the toxic substances was markedly affected by the composition of culture medium, pH, temperature as well as incubation period. Medium of the following composition was favourable for the production of satratoxin H and roridin E : sucrose, 30; $(NH_4)_3PO_4$, 2.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; glutamine, 1.0; $FeSO_4$, 0.01 (g/l, distilled water). Production of both roridin E and satratoxin H was maximal at pH 7 and 30°C, and after incubation period of 12 days for the former and 16 days for the latter.

RÉSUMÉ. — *Stachybotrys atra* (isolat No 128) produit de la satratoxine H et un autre composé toxique, la roridine E. La biosynthèse des deux substances dépend du milieu de culture, du pH, de la température et de la durée d'incubation. Le milieu le plus favorable est composé de : saccharose : 30; $(NH_4)_3PO_4$: 2; KH_2PO_4 : 1; $MgSO_4$: 0,5; KCl : 0,5; glutamine : 1, $FeSO_4$: 0,01 g/litre d'eau distillée. La production des deux substances est maximum à pH 7 et à 30°C, après une incubation de 12 jours pour la roridine E et de 16 jours pour la satratoxine H.

I. — INTRODUCTION

Verrucarins and roridins are macrocyclic diesters and triesters of verrucarol belonging to the trichothecene-type toxins. The substances are metabolites of closely related soil fungi, *Myrothecium roridum* Tode ex Fr. and *Myrothecium verrucaria* (Alb. et Schwein) Ditmer ex Fr. (BAMBURG & STRONG, 1971). Recently EPPLEY et al. (1977) reported production of roridin E, verrucarin J

* Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

Source : MNHN, Paris

and satratoxin H by *Stachybotrys atra*. All trichothecene-type toxins have some biological activities but roridins and verrucarins are the more potent. These compounds are reported to possess antibacterial activity (HARRI et al., 1962), fungistatic properties (LOEFFLER et al., 1964; BAMBURG & STRONG, 1971), insecticidal activity (KISHABA et al., 1962). These compounds, as reported by HARRI et al. (1962) are among the most active cytostatic agents known. In a previous study (EL-KADY & MOUBASHER, 1981), 164 different isolates belonging to *Stachybotrys chartarum* were screened for their respective mycotoxins. Fifty-four isolates among them proved to produce at least one of the following toxins : verrucarol, verrucarine J, roridin E and satratoxin H.

In spite of the accumulating literature of trichothecene-type mycotoxins produced by *Fusarium* fungi, studies on physiological and biochemical aspects of trichothecenes produced by *Stachybotrys* fungi are few. In this study, the role of some factors that influence production of both roridin E and satratoxin H with a locally isolated strain of *Stachybotrys chartarum* was aimed. The isolate used in this study was found to be the most active for production of both roridin E and satratoxin H among varieties of the different isolates tested.

II. — MATERIALS AND METHODS

Organism

Stachybotrys chartarum isolate No 128 was selected from fifty-four toxigenic isolates as a high producer of both satratoxin H and roridin E (EL-KADY & MOUBASHER, 1981).

Cultivation

The fungus was inoculated into autoclaved (121°C, 20 min., 15 lb/in^2) 250-ml Erlenmeyer flasks containing 50 ml of the desired medium. After inoculation with two ml inoculum suspension of two week-old cultures of the pure organism, the flasks were incubated at 28°C, or other mentioned temperatures, for 10 days without shaking.

Preparation of the crude toxin

The method described by UENO et al. (1970), for preparation of *Fusarium* toxin was applied. After incubation, cultures were filtered using vacuum and four layers of cheescloth on Buchner funnel. After washing with 10-15 ml distilled water, the filtrate was mixed with 1% (w/v) of active charcoal, and after standing overnight at 4°C, the charcoal was filtered and immersed in about 10-15 ml of methanol for about 12 h. After filtration of the charcoal, the methanol was evaporated to dryness, and materials remained were referred to as «crude toxin».

Source : MNHN, Paris

Quantitative analysis of the toxins.

The amounts of the toxins were determined by the preparative thin-layer chromatographic technique with authentic samples as controls. A known volume of the methanol eluate was separated using chloroform-methanol (98 : 2, v/v) as solvent system. After development the bands containing the toxins were outlined under long ultraviolet irradiation (365 nm), scraped off, and eluted with methanol. The methanol extract was completed to certain volume with methanol. The exact concentration of each toxin was then determined after making the necessary dilution by ultraviolet spectrophotometric measurement at 262 nm for roridin E and 252 nm for satratoxin H (EPPLEY et al., 1977).

III. – RESULTS

Role of different carbon sources.

Carbon source	Mycelial dry weight (g/100 ml medium)	Roridin E (mg/l)	Satratoxin H (mg/l)
Arabinose	0.073	1.2	1.0
Glucose	0.065	1.6	---
Fructose	0.122	2.2	---
Mannose	0.090	1.6	2.5
Lactose	0.064	1.1	---
Sucrose	0.190	2.4	2.8
Maltose	0.490	2.2	---
Raffinose	0.125	0.9	1.2
Starch	0.495	1.5	1.7
Mannitol	0.308	2.0	1.0
Acetate	0.068	0.5	---
Fumarate	0.115	0.5	0.5
Malate	0.322	---	---
Oxalate	0.386	---	---
Citrate	0.055	---	1.2
Pyruvate	0.500	---	---

Table 1. – Production of satratoxin H, roridin E by *Stachybotrys chartarum* (isolate No 128) as affected by the nature of carbon source.

Source : MNHN, Paris

The present investigation was started by studying the role of carbon sources on the production of both roridin E and satratoxin H by the experimental organism. Czapek's medium of the following composition was used as starting culture medium : glucose, 10; $NaNO_3$, 2; KH_2PO_4, 1; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0,01 (g/l of distilled water). The medium was adjusted to pH 6.5 before sterilization. The cultures were incubated for 10 days at 28°C without shaking. Different carbon sources tested were added as equivalent to 10 gram glucose per liter. Table 1, shows that the different carbon sources supported the growth of the fungus with varying degrees, very weak growth was observed when pyruvate, citrate, acetate, lactose, glucose, arabinose or mannose was used as the sole carbon source. Best mycelial growth was obtained using starch or maltose followed by oxalate, malate or mannitol, respectively. Although oxalate and malate supported fairly good growth, yet none of them was suitable for detectable amount of either satratoxin H or roridin E. In case of glucose, fructose, lactose, maltose or acetate, only roridin E was detected. Also in case of citrate only satratoxin H was detected as a single and final product. Both satratoxin H and roridin E were detected when arabinose, mannose, sucrose, raffinose, starch, mannitol and fumarate were used as carbon sources. However, sucrose proved to be superior, since it supported the highest yield of satratoxin H and roridin E.

Variation of sucrose concentration affected both mycelial growth and toxin production. Maximum production of satratoxin H (4.6 mg/l), roridin E (3.1mg/l) and fungal growth (0.310 g/100 ml medium) were obtained at 30, 50 and 50 g/l of sucrose concentration respectively.

Role of different nitrogen sources.

Czapek's medium with sucrose (30 g/l) as carbon source was used during this study. The different nitrogen sources were added as nitrogen equivalent to 2 g/l of $NaNO_3$. As shown in Table 2, both ammonium nitrogen and nitrate nitrogen were utilizable and suitable for mycelial growth and toxins production, but $Ca(NO_3)_2$ supported good mycelial growth with no toxins production. Also $NaNO_2$ and yeast extract supported a good mycelial growth only with no toxins produced. Peptone, ammonium sulphate and ammonium chloride were favourable for mycelial growth and roridin E production. On the other hand, ammonium phosphate was the best nitrogen source for both roridin E and satratoxin H production.

Elevation of nitrogen level between 0.5 and 2.0 g/l of ammonium phosphate, exerted a rise in the production of roridin E and satratoxin H (2.8 to 5.1 mg/l and 3.1 to 5.5 mg/l, respectively) followed by a sharp decrease in the yields of both toxins between 3 and 6 g/l of ammonium phosphate. However, growth increased regularly with the increase of nitrogen concentration reaching its maximum at 4 g/l (0. 384 g/l), which followed by a slight decrease at 6.0 g/l (0.262 g/l).

Source : MNHN, Paris

Nitrogen source	Mycelial dry weight (g/100 ml medium)	Roridin E (mg/l)	Satratoxin H (mg/l)
Control (nitrogen source omited)	0.094	---	---
$NaNO_3$	0.255	3.4	4.5
KNO_3	0.145	3.6	4.4
$Ca(NO_3)_2$	0.220	---	---
NH_4NO_3	0.135	2.2	3.1
$NaNO_2$	0.156	---	---
NH_4Cl	0.174	3.2	---
$(NH_4)_2SO_4$	0.166	3.8	---
$(NH_4)_3PO_4$	0.292	4.8	5.1
Yeast extract	0.308	---	---
Peptone	0.350	2.8	---

Table 2. — Production of satratoxin H and roridin E by *Stachybotrys chartarum* (isolate No 128) as affected by the type of nitrogen source.

Effect of hydrogen ion concentration.

Modified medium of sucrose (30 g/l) and ammonium phosphate (2 g/l) as carbon and nitrogen sources were used during this study. The results of Table 3 reveal the detection of a new trichothecene-type substance, when *S. chartarum* was grown at pH values ranging from 7.5 to 10. This substance was identified by thin-layer chromatographic analysis as verrucarol (fig. 1). Concentration of satratoxin H increased with the increase of pH value, reaching maximum at pH 7, then gradually diminished. Roridin E production also increased gradually reaching maximum at pH 7, followed by a sharp decline which was associated with the appearance of verrucarol which had its maximum yield at pH 9.

Source : MNHN, Paris

pH value	Mycelial dry weight (g/100 ml medium)	Roridin E (mg/l)	Satratoxin H (mg/l)	Verrucarol
4.0	---	---	—	---
4.5	0.095	---	2.1	---
5.0	0.110	0.5	4.0	—
5.5	0.122	2.1	4.2	---
6.0	0.156	4.0	4.8	—
6.5	0.285	5.1	5.3	---
7.0	0.340	5.4	5.9	---
7.5	0.380	2.4	3.4	+
8.0	0.390	1.8	2.0	++
8.5	0.390	1.6	1.8	+++
9.0	0.360	1.6	1.5	++++
9.5	0.310	---	---	+++
10.0	0.280	—	---	+

Table 3. — Production of roridin E and satratoxin H by *Stachybotrys chartarum* (isolate No 128) as affected by variation of pH values of the culture medium.

Role of amino acids and derivatives.

As shown in Table 4, all amino acids and derivatives tested promoted mycelial growth (with the exception of cysteine which retarded both mycelial growth and toxins production). However, most of the amino acids did not noticeably affect toxins production. Glutamic acid was the best amino acid for mycelial growth, while glutamine was the most suitable for roridin E and satratoxin H production (7.6 and 6.2 mg/l, respectively).

Effect of incubation temperature.

The fungal growth and toxins production were investigated at different temperatures ranging from 5 to 45°C. Mycelial growth, roridin E and satratoxin H formation increased parallel with the increase of incubation temperature reaching maxima at 30°C (1.47 g/l, 7.5 mg/l and 6.3 mg/l, respectively). With

Source : MNHN, Paris

Amino acids (1g/1)	Mycelial dry weight (g/100 ml medium)	Roridin E (mg/1)	Satratoxin H (mg/1)
Control (without amino acids)	0.360	5.6	5.9
Lysine	0.920	7.2	4.2
Asparagine	0.900	5.7	3.3
Serine	0.688	6.2	4.2
Glycine	0.852	5.5	3.4
Arginine	0.980	5.7	5.8
Leucine	0.925	7.0	5.8
Valine	0.712	7.4	4.9
Norvaline	0.685	4.3	3.7
Alanine	0.828	6.1	4.3
Phenylalanine	0.746	6.8	4.5
Glutamic acid	1.484	6.2	4.4
Glutamine	1.388	7.6	6.2
Cysteine	0.308	4.1	1.2

Table 4. – Production of roridin E and satratoxin H by *Stachybotrys chartarum* (isolate No 128) as affected by the addition of some amino acids.

the elevation of temperature, mycelial growth as well as toxins production decreased sharply. No growth was detected at 5°C and 45°C.

Effect of incubation period.

Data presented in Table 5 show mycelial growth and toxins production of *S. chartarum* when grown for different time intervals on the modified culture medium adjusted to pH 7 at 30°C. Under these conditions, the mycelial growth increased as the culture aged, reaching maximum after ten days, followed by a decline with the lengthening of incubation period. A maximum concentration of roridin E was recorded after 12 days of incubation. Thereafter, the concen-

Source : MNHN, Paris

Incubation period (days)	Mycelial dry weight (g/100 ml medium)	Roridin E (mg/l)	Satratoxin H (mg/l)
2	0.035	0.7	---
4	0.246	2.9	0.5
6	0.464	4.1	1.9
8	1.200	5.2	2.8
10	2.020	6.7	4.2
12	1.875	8.9	4.5
14	1.612	7.3	5.6
16	1.274	5.4	7.6
18	0.724	3.1	4.8
20	0.226	1.7	2.4

Table 5. — Production of roridin E and satratoxin H by *Stachybotrys chartarum* (isolate No 128) at various incubation periods.

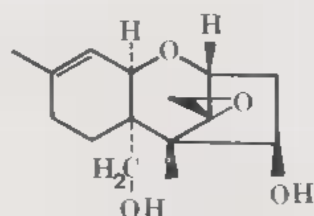
tration declined regularly with the lengthening of incubation period so that less than 20% of the maximum was estimated after 20 days of incubation. Satratoxin H was not detected before four days of incubation, and its concentration regularly increased with the increase of incubation period, so that the maximum was reached after 16 days of incubation. After longer periods it sharply fell off so that only 31 % of the maximum was estimated after 20 days.

IV. — DISCUSSION

There were noticeable differences in both mycelial growth and toxins production with different carbon sources tested. Carbon sources affected growth or toxins production independently. For example, malate and oxalate supported fairly good mycelial growth, but failed to support any detectable amount of either satratoxin H or roridin E. On the reverse, arabinose, glucose, mannose or citrate supported toxins production rather than mycelial growth. Using glucose, fructose, mannose, lactose or acetate, only roridin E was detected as single

Source : MNHN, Paris

and final end product, which means that these compounds are unfavourable for the bioconversion of roridin E to satratoxin H. Recently, EPPLEY et al. (1977) proposed that roridin E is the precursor of satratoxin H and the former is transformed to the latter by two biological steps, addition of a hydroxyl group on C-12' and dehydrogenative ring formation between C-6' and C-12' of roridin E, (fig. 1). Using citrate as the sole carbon source, only satratoxin H was detected and this means that citrate stimulated the enzymatic system(s) responsible for the transformation of roridin E to the final end product satratoxin H. Increasing the initial concentration of sucrose resulted in a regular increase of both fungal growth and satratoxin H biosynthesis, reaching maxima at 50 [illegible] and 30 g/l respectively. However, roridin E production was more or less unaffected by variation of sucrose concentration.



Verrucarol

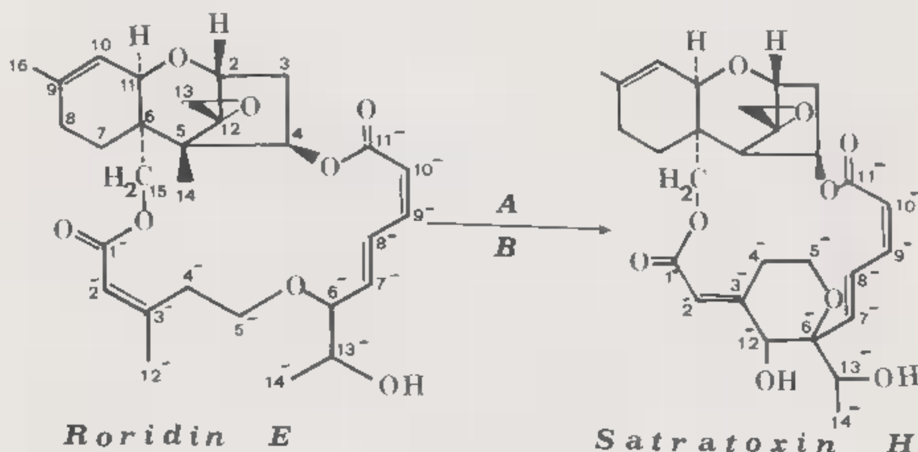


Fig. 1. – Chemical structure of verrucarol and the proposed biological steps for the transformation of roridin E to satratoxin H. A : Addition of a hydroxyl group on C-12'. B : Dehydrogenative ring formation between C-6' and C-12' of roridin E.

Source : MNHN, Paris

Ammonium nitrogen and nitrate nitrogen were suitable for mycelial growth as well as for roridin E production but with various degrees. However $Ca(NO_3)_2$ supported fungal growth rather than toxin formation. Maximum yields of roridin E and satratoxin H were obtained using $NaNO_3$, KNO_3 and $(NH_4)_3PO_4$, but ammonium phosphate was superior.

Reports on trichothecenes production by *Stachybotrys* fungi are few to make a conclusion as to the condition favourable for such production. However, the best carbon sources for *Fusarium poae* and *Fusarium sporotrichioides*, reported as trichothecene-type mycotoxin producers, were starch and glucose. Best results among inorganic substances were obtained with ammonium sulphate and sodium nitrate (JOFFE, 1971). According to BILAJ & PIDOPLICHKO (1970) the following carbohydrates favoured toxin production by *S. alternans* (toxin not identified) : arabinose, galactose, starch, glucose, sucrose, maltose, raffinose, cellobiose and dextrin.

Using ammonium phosphate as nitrogen source, variation of the nitrogen level of the culture medium between 0.5-2.0 g/l, induced an increase in the formation of both roridin E and satratoxin H, followed by a sharp decline at higher levels (3-6 g/l).

Since $(NH_4)_3PO_4$ was recorded superior than any other ammonium nitrogen sources tested (ammonium sulphate and ammonium chloride) it is thus possible that the phosphate radicle played an important role in toxin biosynthesis. In this respect ACHILLADELIS & HANSON (1967), and ACHILLADELIS et al. (1970), reported incorporation of mevalonic acid and farnesol pyrophosphate into trichothecene-type mycotoxins (trichodermol, trichothecin and trichothecolone) which indicates that a 6,7-trans-farnesol pyrophosphate is a precursor of these compounds.

Variation of pH of the culture medium affected toxins production in a different manner. Satratoxin H increased with the increase of pH values reaching maximum at pH 7. This indicated that the optimal pH for the bioconversion of roridin E to the end product satratoxin H is around neutrality. Also roridin E production reached maximum 5.4 mg/l at pH 7, followed by a sharp decrease at higher pH values, which was associated with the appearance of verrucarol. As has been established, all roridins and verrucarins are macrocyclic esters of 4,15-dihydroxy-12,13-epoxy-Δ^9-trichothecene (verrucarol). All roridins are formed via the ester formation between the free hydroxyl groups of verrucarol (C-4 and C-15) and the dicarboxylic groups of roridinic acid (or derivatives) (BOHNER & TAMM, 1966; ZURCHER & TAMM, 1966). Two postulates may be put forward for explaining the decrease of roridin E accompanied by increase and accumulation of verrucarol at high pH values. Firstly the chemical hydrolysis of the macrocyclic is enhanced at high pH values leading to the accumulation of verrucarol, which presumably proceeds during fungal growth. The second is that the enzymatic diester formation achieved between verrucarol and roridinic acid derivative is retarded at alkaline pH.

All amino acids and derivatives tested (except cysteine) promoted mycelial

Source : MNHN, Paris

growth but almost with no qualitative differences in toxins production. Serine, leucine, alanine, phenylalanine, valine and glutamine promoted the formation of toxins especially roridin E. UENO et al. (1970) using a strain of *Fusarium nivale* (Fn-2B), reported that among the amino acids tested, phenylalanine, tryptophan and methionine, promoted the formation of fusarenone-x, a cytotoxic mycotoxin of trichothecene -type. BILAJ, (1953) stated that aspartic acid, glutamic acid and their amides, alanine and glycol, all provide suitable source of nitrogen for toxin production by *Fusarium* species of the section Sporotrichiella; the toxin was identified later as trichothecene-type mycotoxin (PALTI, 1978).

Incubation temperature proved to be an important factor in toxin production by the experimental organism. 30°C was the optimum for both mycelial growth and toxins production. BOHNER et al. (1965) reported successful production of roridin E (2.2 mg/l) when *Myrothecium roridum* was cultivated at 27°C in submerged culture. KORPINEN et al. (1974), reported the production of cytotoxic toxins (toxins not identified) by *Stachybotrys alternans* grown on Czapek's medium supplemented with thiamine, and nicotinic acid and incubated at 22°C for 4-6 weeks.

The time of harvesting of the culture medium is a decisive factor of the production of both satratoxin H and roridin E. The yield of roridin E reached maximum after 12 days of incubation and of satratoxin H after 16 days. The delay of satratoxin H maximum production four days later than that of roridin E is reasonable since satratoxin H is derived from roridin E as shown in fig. 1, (EPPLEY et al., 1977).

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BIBLIOGRAPHIE

ACHILLADELIS B. & HANSON J.R., 1967 — The role of farnesyl pyrophosphate in the biosynthesis of trichothecin. *Chem. & Ind.* (London), 1643.

ACHILLADELIS B., ADAMS P.M. & HANSON J.R., 1972 — Studies in terpenoid biosynthesis. Part VIII. The formation of trichothecane nucleus. *J. Chem. Soc. Perkin* I, 1425-1428.

BAMBURG J.R. & STRONG F.M., 1971 — 12,13-Epoxytrichothecenes. p. 207-292, in KADIS S., CIEGLER A. & AJL S.J. (Eds.) : Microbial Toxins, Vol. VII, Academic Press, New York.

BILAJ, V.I., 1953 — Toxic fungi on grain of cereal crops. p. 1-93, *Publ. Acad. Sci. Ukr.*

Source : MNHN, Paris

SSR Kiev. (Russ.).

BILAJ V.I. & PIDOPLICHKO N.M., 1970 Toxicogenic microscopical fungi. *Acad. Nauk Ukr. SSR, Naukova Dumka, Kiev.* 132-145 (Russ.).

BOHNER B. & TAMM C., 1966 – Die konstitution von roridin A. *Helv. Chim. Acta.* 49 : 2527.

BOHNER B., FETZ E., HARRI E., SIGG H.P., STOLL C. & TAMM C., 1965 – Uber die isolierung von verrucarin H, verrucarin J, roridin D und roridin E aus *Myrothecium*-Arten. *Helv. Chim. Acta.* 48 : 1079-1087.

EL-KADY I.A. & MOUBASHER M.H., 1981 – Toxigenicity and toxins of *Stachybotrys* isolates from wheat samples in Egypt. *Experimental Mycology* 5 : (in press).

EPPLEY R.M. & BAILEY W.J., 1973 – 12,13-Epoxy-Δ^9-trichothecenes as the probable mycotoxins responsible for stachybotryotoxicosis. *Science* 181 : 758-760.

EPPLEY R.M., MAZZOLA E.P., HIGHET R.T. & BAILEY W.J., 1977 – Structure of satratoxin H, a metabolite of *Stachybotrys atra*. Application of proton and carbon-13 nuclear magnetic resonance. *J. Org. Chem.* 42 : 240-243.

HARRI E., LOEFFLER W., SIGG H.P., STAHELIN H., STOLL C., TAMM C. & WIESINGER D., 1962 – Uber die verrucarine und roridine, eine gruppe von cytostatisch hock-wirksamen antibiotica aus *Myrothecium*-Arten. *Helv. Chim. Acta.* 45 : 839-853.

JOFFE A.Z., 1971 – Alimentary toxic aleukia, *in* KADIS S., CIEGLER A. & AJL S. (Eds.) : Microbial Toxins, p. 137-189, vol. VIII, Academic Press, New York.

KISHABA A.N., SHANKLAND D.L., CURTIS R.W. & WILSON M.C., 1962 – *J. Econ. Entomol.* 55 : 211. Cited in BAMBURG J.R. & STRONG F.M., 1971.

KORPINEN E.L., KURKINENE M., NUMMI M. & ENARI T.M., 1974 – Studies on *Stachybotrys alternans.* III. Chromatographic separation and tissue culture toxicity test of stachybotryotoxins. *Acta Pathol. Microbiol. Scand., Section B*, 82 : 7-11.

LOEFFLER W., MAULI R., RUSCHE M.E. & STAHELIN H., 1964 – French Patent, 1, 372, 122; *Chem. Abstract* 1965-62 : 5856 d.

PALTI J., 1978 – Toxigenic *Fusaria*, their distribution and significance as cause of disease in animal and man. Verlag Paul Parey (Publ.) Berlin und Hamburg. 110 p.

UENO Y., ISHIKAWA Y., SAITO-AMAKAI K. & TSUNODA H., 1970 – Environmental factors influencing the production of fusarenon-x, a cytotoxic mycotoxin of *Fusarium nivale* Fn-2B. *Chem. Pharm. Bull.* 18 : 304-312.

ZURCHER W. & TAMM C., 1966 – Isolierung von 2'-dehydroverrucarin A als metabolit von *Myrothecium roridum* Tode ex Fr. Gattungstypus Fries. *Helv. Chim. Acta.* 49 : 2594.

Source : MNHN, Paris